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Short Communication

Bone Marrow–Derived Progenitor Cells Do Not Contribute to Podocyte Turnover in the Puromycin Aminoglycoside and Renal Ablation Models in Rats

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A key event in the progression of glomerular disease is podocyte loss that leads to focal and segmental glomerulosclerosis (FSGS). Because adult podocytes are postmitotic cells, podocyte replacement by bone marrow–derived progenitors could prevent podocytopenia and FSGS. This study uses double immunofluorescence for Wilms' tumor-1 and enhanced green fluorescent protein (eGFP) to examine whether an eGFP-positive bone marrow transplant can replace podocytes under normal circumstances and in 3 different rat models of FSGS: puromycin aminoglycoside nephropathy, subtotal nephrectomy, and uninephrectomy. Bone marrow engraftment was successful, with more than 70% eGFP-positive cells and virtually normal histologic findings. No bone marrow transplant–derived podocytes were found in four control rats after transplantation, in nine rats at up to 10 weeks after puromycin aminoglycoside nephropathy induction, in three rats 23 days after subtotal nephrectomy, and in six rats up to 21 days after uninephrectomy. A total of 2200 glomeruli with 14,474 podocytes were evaluated in all groups. Thus, podocyte replacement by bone marrow–derived cells does not contribute to podocyte turnover in rats, even in models of podocyte damage. This is in contrast to previous studies in mice, in which bone marrow–derived podocytes were found. Further studies will address this discrepancy, which could be explained by species differences or by predominant podocyte regeneration from a parietal epithelial cell niche. ***(Am J Pathol 2011, 178:494–499; DOI: 10.1016/j.ajpath.2010.10.024)***

Adult podocytes are considered postmitotic cells virtually incapable of replication and do not proliferate to a measurable extent even in subtotal nephrectomy.[1–9] The exceptions are possibly diseases such as the collapsing variant of focal and segmental glomerulosclerosis (FSGS) or crescentic glomerulonephritis.[10] Podocyte damage leading via podocytopenia to FSGS is thought to play a pivotal role in the progression of glomerular diseases to FSGS.[2,11,12] This has been documented in human IgA glomerulonephritis,[13] hypertensive glomerulopathy,[14] diabetic nephropathy,[15,16] and animal models of transplant glomerulopathy,[17] and membranous[18] and immune complex glomerulonephritis.[19]

Therefore, a considerable research effort has been undertaken recently to examine podocyte replacement by extrarenal and also intrarenal progenitors. Several groups have reported podocyte replacement by bone marrow–derived progenitor cells in mice receiving bone marrow transplants,[20] mouse models of diffuse mesangial sclerosis,[21] and mouse models of Alport's syndrome.[22,23] Canine mesenchymal stem cells were shown

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to express the podocyte marker proteins synaptopodin and CD2AP *in vitro* when seeded on components of the glomerular basement membrane (NC1 hexamers of collagen type IV).[24] Recipient-derived progenitor cells seem to contribute to podocyte turnover in human renal transplants.[25] Also the recent finding of potential intrarenal podocyte progenitors lining Bowman's capsule in mice[26] and humans[27,28] has stirred great interest. For therapeutic purposes, bone marrow–derived progenitors would offer more potential than intrarenal progenitors because they can more easily be manipulated for additional therapeutic benefits.

Therefore, the potential of podocyte replacement by bone marrow–derived progenitors is examined in three different rat models for the present study: puromycin aminoglycoside nephropathy (PAN), subtotal nephrectomy (SN), and uninephrectomy (UN). Puromycin aminoglycoside is considered a selective podocyte toxin. Its exact mechanism of action is not known, but reactive oxygen species are considered to be contributory.[29,30] Podocyte loss then leads to FSGS. SN causes compensatory glomerular hypertrophy with secondary podocyte damage and ensuing FSGS.[1,31,32] UN is a milder model of glomerular damage, that, dependent on the age, strain, feeding, and survival time of the animal, may[33] or may not show FSGS.[34]

To track potential podocyte progenitors from the bone marrow, PAN and SN were induced in rats after transplantation of bone marrow from enhanced green fluorescent protein (eGFP) transgenic rats. Although the autofluorescence of eGFP is lost on paraffin embedding, it can easily be visualized by immunofluorescence (IF) and serves as a permanent marker of the transplanted bone marrow cells and their progeny, even after differentiation.[35,36] By double IF staining for the specific nuclear podocyte marker Wilms' tumor-1 (WT1)[37] and cytoplasmic eGFP, bone marrow transplant–derived podocytes can be identified. Thus, the rate at which bone marrow–derived cells contribute to podocyte turnover in health and the three disease models, one with primary podocyte damage and two with severe and milder secondary podocyte damage, can be calculated.

Materials and Methods

Animal Procedures

After lethal total body irradiation (9 Gy), female Wistar rats (body weight, 60 to 80 g) were given bone marrow transplants containing 2×10^6 cells from eGFP-positive male Wistar rats 24 hours later. eGFP-positive transgenic rats have been previously described in detail.[35]

Eight weeks after bone marrow transplantation, PAN, SN, and UN were induced. For PAN, a single shot of 15 mg of puromycin aminoglycoside (P7130; Sigma-Aldrich, Seelze, Germany) per 100 g of body weight was given intravenously. Three rats for each time point were examined at 15 and 23 days and after 10 weeks.

SN was performed in three rats as previously described in detail.[38] In brief, two-thirds of the left kidneys were removed by ligation followed by a right-sided nephrectomy 7 days later through flank incisions. All animals underwent necropsy at 23 days after nephrectomy of the right kidney.

Six rats were uninephrectomized; two were analyzed after 7 days, two after 10 days, and two after 21 days. Four rats grafted with eGFP-positive bone marrow served as controls; one was sacrificed at day 15, one at 23 days, and two at 10 weeks.

All animals were kept in standard housing with water and standard chow *ad libitum*.

Fluorescence-Activated Cell Sorting Analysis of Peripheral Blood

Eight weeks after the bone marrow transplantation, the peripheral blood was analyzed for donor chimerism by fluorescence-activated cell sorting. Using a FACScan (Becton Dickinson, Franklin Lakes, NJ) with CellQuest software (BD Biosciences, San Jose, CA), the eGFP-positive leukocyte repopulation was analyzed from 200 μL of heparinized peripheral blood after removal of erythrocytes with Erythrocyteolysis Buffer (Becton Dickinson). Only fully reconstituted rats with a peripheral blood donor chimerism of more than 90% were included in the studies.

Paraffin and Ultrastructural Histology, Glomerular Morphometry, and Double IF

Organs were perfusion fixed with formalin and embedded in paraffin for light microscopy and Epon for transmission electron microscopy. For conventional light microscopy, 3-μm paraffin sections of kidney tissue were stained with PAS. Vertebral bone sections were Giemsa stained after decalcification in EDTA.

Glomerular volume, podocyte volume density, and mean podocyte numbers per glomerulus were determined on WT1-immunostained thick and thin sections as recently described.[39] In brief, WT1-positive podocyte nuclei were counted on 30 glomerular sections each of 8 μm and 4 μm. Measurements of the glomerular cross-sectional area served to determine glomerular volume and podocyte volume density. Glomerular volume and podocyte volume density then yielded mean podocyte numbers per glomerulus.

Segmental glomerulosclerosis (SGS) and global glomerulosclerosis (GGS) were quantified on PAS-stained slides of each specimen by examination of 300 glomeruli that were chosen in a serpentine movement from outer to inner cortex and back. For ultrastructural examination, kidney tissue was postfixed and contrasted in osmium tetroxide, embedded in Epon, and contrasted with lead citrate and uranyl acetate. Ultrathin sections were evaluated on a transmission electron microscope (EM 10; Zeiss, Oberkochen, Germany).

For double IF, 3-μm sections were dewaxed in xylol and graded ethanols. Antigens were retrieved in Tris/EDTA buffer (10 mmol/L Tris base, 1 mmol/L EDTA solution, 0.05% Tween 20, pH 9.0) at 90°C for 25 minutes.

After blocking with diluted donkey serum (no. 017-000-121; Jackson ImmunoResearch Laboratories, West Grove, PA), mouse monoclonal anti-WT1 (NCL-L-WT1-562; Novocastra, Newcastle Upon Tyne, United Kingdom) and rabbit polyclonal anti-GFP (ab290; Abcam, Cambridge, United Kingdom) were used as primary antibodies. WT1 was visualized in red with Cy3-labeled secondary anti-mouse antibodies (no. 715-166-151; Jackson ImmunoResearch Laboratories) and eGFP in green with Cy2-labeled secondary anti-rabbit antibodies (no. 711-226-152; Jackson ImmunoResearch Laboratories). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole by mounting with Immunoselect Antifading Mounting Medium (Dianova, Hamburg, Germany). Kidney and bone marrow tissue from eGFP-positive and eGFP-negative rats served as controls. The sensitivity for the detection of eGFP bone marrow–derived podocytes was assumed to be on average approximately 75%, calculated as the number of WT1-positive podocyte nuclei surrounded by eGFP-positive cytoplasm divided by the number of WT1-positive podocyte nuclei. The specificity should approach 100% because WT1 is considered a specific marker for podocytes in the glomerular tuft[37] and because no cells stained eGFP positive in wild-type control rats. One hundred glomeruli from each rat were examined on these double-stained sections for the presence of bone marrow transplant–derived, WT1-positive, eGFP-positive podocytes.

Statistical Analyses

Calculations and statistical analyses were performed with JMP (SAS Institute Inc, Cary, NC) on a Macintosh System (Apple, Cupertino, CA). For comparison of continuous variables, Wilcoxon tests were used. Differences were regarded as significant with $P < 0.05$ in two-sided tests.

Ethical Approval

All experiments have been approved by the ethics committee of the University Hospital Hamburg-Eppendorf, Hamburg, Germany.

Results

Fluorescence-Activated Cell Sorting Analysis of Peripheral Blood

The peripheral blood of each rat who received an eGFP bone marrow transplant was examined for donor chimerism by FACScan (Becton Dickinson) (Figure 1) and compared with the peripheral blood of normal rats not receiving transplants by overlay. Most rats exhibited more than 90% eGFP-positive leukocytes, indicative of a complete bone marrow reconstitution.

Bone Marrow Histology

To document proper engraftment of the bone marrow transplants, vertebral bone marrow from all animals was examined on eGFP immunostained and Giemsa slides by an expert hematopathologist (G.B.). At least 70% of the hematopoietic cells in the bone marrow of the rats receiving transplants were immunohistochemically eGFP positive. Bone marrow histologic findings were virtually normal on Giemsa-stained slides, showing only slight alterations in granulopoiesis and slight hyperplasia of megakaryopoiesis in a few cases.

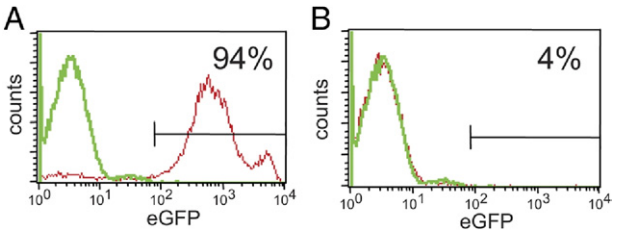

Figure 1. Overlay of FACScan analyses of peripheral blood from wild-type and female Wistar rats receiving eGFP bone marrow transplants 8 weeks after transplantation. **A:** Successful bone marrow transplantation with 94% donor chimerism (red graph) in eGFP transplanted rats compared with wild-type rats (green graph). **B:** Unsuccessful bone marrow transplantation with only 4% donor chimerism (red graph). This rat was not included for further studies.

Renal Histology

All control kidneys showed virtually normal renal histologic findings with only minimal SGS (see as follows) and otherwise unremarkable glomeruli, tubules, and vessels on light microscopic and ultrastructural examination.

Animals in the PAN cohort showed FSGS, focal glomerulosclerosis, and GGS at all time points examined (Figure 2). Sclerotic glomerular segments initially displayed a foamy, loose matrix; later these segments had a more hyalinotic aspect. Increasing tubular atrophy and interstitial fibrosis and hyalinotic obliteration of arterioles was found throughout the observation period. Ultrastructural examination revealed foot process effacement of up to 50% and villous projections of podocytes and segmentally swollen endothelium on day 15. On day 23 and in week 10 changes were less marked, with foot process effacement below 15%, few villi, and unremarkable endothelium.

In the SN cohort, FSGS, focal glomerulosclerosis, and GGS were evident (Figure 1). At 23 days after SN, sclerotic glomerular segments also had a loose foamy matrix. Glomerular hyalinosis was not noted in SN. Foot process effacement was approximately 10%; podocytes had few cytoplasmic villi. Few glomerular endothelial cells were lightly swollen.

All kidneys in the UN cohort had an almost normal histologic appearance of vessels, glomeruli, and tubuli at days 7 and 10. At day 21 slight focal and segmental expansion of the mesangial matrix was apparent. Ultrastructural finding in the glomeruli were unremarkable; podocytes had well-preserved foot processes.

Quantification of SGS and GGS

SGS and GGS were quantified in 300 glomeruli from each specimen; the results are given in Table 1. In the control

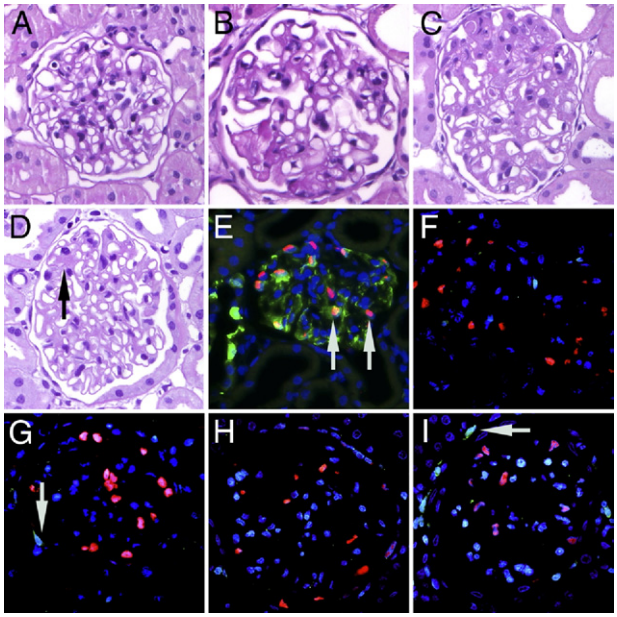

Figure 2. Normal glomerulus in a control rat 10 weeks after the eGFP bone marrow transplantation (**A**); PAN at day 15 with sclerotic glomerular segment at 5 to 8 o'clock with hyalinotic, loose matrix (**B**); SN at week 4 with early segmental sclerosis between 11 and 2 O'clock (**C**); glomerulus from the UN cohort (**D**) 21 days after removal of the contralateral kidney with slight mesangial expansion (**arrow**). **A–D:** PAS, original magnification ×400. **E:** eGFP-positive rat as a positive control for double IF stain, labeling WT1-positive podocyte nuclei red and eGFP green. All podocytes are WT1 positive; on average approximately 75% were also positive for eGFP (**left arrow**). In some podocytes eGFP expression could not be detected by IF (**right arrow**). **F:** Control rat 4 weeks after eGFP bone marrow transplantation; none of the podocytes with the nuclear WT1 positivity (red) showed cytoplasmic staining for eGFP, which would indicate derivation from the bone marrow transplant. **G:** PAN rat at day 15, again no WT1-positive, eGFP-positive cells, equivalent of bone marrow transplant–derived podocytes. Note pericapsular eGFP-positive cell (**arrow**). **H:** SN rat at 4 weeks; only WT1-positive podocytes without eGFP immunoreactivity. **I:** UN rat at day 7; again no bone marrow transplant–derived, WT1-positive, eGFP-positive podocytes but pericapsular eGFP-positive bone marrow transplant–derived cell (**arrow**) as internal control. **E–I:** Double IF staining WT1 in red and eGFP in green with DAPI nuclear counterstain (blue) (original magnification ×400 for all).

rats, SGS was present in a mean ± SD of 0.3% ± 0.47% of the glomeruli and GGS was not found (0% ± 0%). In the combined PAN cohort, SGS was present in 10.1% ± 6.98% of the glomeruli and GGS in 1.1% ± 2.23%. Only SGS was significantly more prevalent than in the control cohort ($P = 0.0294$). In the SN cohort, the frequency of SGS was 5.8% ± 2.69% and the frequency of GGS was 0.8% ± 0.51%, both significantly more prevalent than in the controls ($P = 0.0477$ for SGS and $P = 0.0319$ for GGS). In the combined UN cohort, SGS was present in 0.1% ± 0.14% of glomeruli and GGS in 0% ± 0%. Thus, both SGS and GGS were not more prevalent in the combined UN than in the control cohort.

Glomerular Morphometry

The mean podocyte number per glomerulus was 79 ± 37 in the combined subgroups of the PAN cohort, 192 ± 62 in the SN cohort, 148 ± 45 in the combined UN cohorts, and 155 ± 62 in the combined controls. Thus, podocyte numbers per glomerulus were lower in the PAN cohort than in the controls ($P = 0.0253$), whereas there was no difference between the other cohorts and the controls.

The mean glomerular volume was $1.05^6 \pm 0.314^6$ μm^3 in the combined subgroups from the PAN cohorts, $1.16^6 \pm 0.400^6$ μm^3 in the SN cohort, and 1.30 ± 0.123^6 μm^3 in the combined subgroups of the UN cohort, whereas it was $1.37^6 \pm 0.252^6$ in the controls. Thus, no differences could be found among the PAN, SN, and UN cohorts and the controls.

Double Immunofluorescence Staining

In none of the animals in the four cohorts examined (PAN, SN, UN, and controls) WT1-positive, eGFP-positive glomerular cells, equivalent to eGFP-positive bone marrow transplant–derived podocytes, were found (Figure 1). In the four control rats, a total of 400 glomeruli containing 2747 podocytes were evaluated. In the PAN group, 900 glomeruli with 5833 podocytes were examined. In the SN group, 300 glomeruli with 1563 podocytes were examined. In the UN group, 600 glomeruli with 4331 podocytes were examined.

Discussion

The present experiments are the first, to our knowledge, to explore the potential role of bone marrow–derived progenitor cells in the replacement of podocytes after the induction of podocyte damage in rats. In two of the disease models, PAN and SN, podocyte damage with foot process effacement and villous transformation with subsequent FSGS, in SN and GGS, was successfully induced as evident by histologic examination. Despite regular engraftment of eGFP-positive bone marrow at a rate of more than 70% of the nucleated cells and fluorescence-activated cell sorting-proven chimerism in the peripheral blood, no podocytes deriving from the eGFP-positive bone marrow precursors could be found in a total of 2200 glomeruli with 14,474 podocytes examined in the FSGS models, the UN model, and the controls. The present model system of eGFP transgenic rats is limited by the mosaic expression of the vector because expression of eGFP could be detected by IF in only approxi-

Table. Cohorts and Frequency of Segmental SGS and GGS

Cohort	Δt	No.	SGS, mean ± SD, %	GGS
Controls	15 days	1	0.3 ± 0.47	0 ± 0
	23 days	1		
	10 weeks	2		
PAN	15 days	3	10.1 ± 6.98*	1.1 ± 2.23
	23 days	3		
	10 weeks	3		
SN	23 days	3	5.8 ± 2.69*	0.8 ± 0.51*
UN	7 days	2	0.1 ± 0.14	0 ± 0
	10 days	2		
	21 days	2		

Δt, time after induction of PAN, SN, UN or time after bone marrow transplant in control animals.
*$P < 0.05$ when compared with the combined control cohort in two-sided Wilcoxon tests.

mately 75% of the podocytes and even less of tubular epithelial cells. Still, the relatively large number of podocytes examined in this study should overcome this limitation. Therefore, it seems safe to draw the conclusion that podocyte replacement by bone marrow–derived cells after initial podocyte injury or as contribution to a continuous normal turnover does not occur in the present rat models.

These results should be interpreted with caution. Because the present results from Wistar rats are in contrast to data in which bone marrow transplant–derived podocytes were found in wild-type mice[20] and genetic mouse models with diffuse mesangial sclerosis[21] and Alport's syndrome[22,23] in a frequency of approximately 10% at 13 weeks after transplantation, the possibility of podocyte replacement by bone marrow–derived cells should be further examined in other model systems.[23] In addition, the finding that bone marrow–derived podocytes have also been demonstrated in healthy control mice at a rate of approximately 0.7% 4 and 12 weeks after bone marrow transplantation[21] is a further indication of the need for additional studies in this area.

Currently, these discrepant results cannot be definitely explained but may be due to species differences and/or different progenitor niches for podocyte regeneration. The alternative progenitor niche for podocyte regeneration might be in the parietal epithelial cell layer as recently described in mice[26] and humans.[27,28] This niche has not yet been characterized in rats. The differences might also be due to the underlying disease because experiments in mice used genetic variants of kidney diseases, which might also involve different progenitor cell niches and different chemokine receptor and ligand expression for attraction of these progenitors.

Our experiments demonstrate that disease models that lead via primary (PAN) or secondary (SN) podocyte damage to FSGS are not associated with immigration and differentiation into podocyte lineage of bone marrow–derived progenitors. Of course, this does not exclude that passenger progenitors might be present during the disease and affect FSGS development through paracrine mechanisms. Further studies should examine which niche, bone marrow–derived, or parietal epithelial cell layer contributes to what extent to podocyte regeneration and what factors could foster this process to develop therapeutic strategies for podocyte replacement in diseased glomeruli.

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